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13. ABSTRACT *(Maximum 200 Words)*
Our research goals involve identifying and characterizing proteins/peptide targeting sequences that could be used in the specific delivery of chemotherapeutic agents to breast cancer cells. In order to achieve these goals, we have developed a novel targeting system based on a genetic screen using a modified, multifunctional λ bacteriophage vector. In the first year, we have developed, optimized and validated several different components of this bacteriophage display system. First, we confirmed the integrity of our test constructs, and produced both DNA and sterile bacteriophage-expressing fusion proteins on the capsid surface for internalization studies. Second, we optimized the recovery of the integrated bacteriophage DNA containing the plasmid allowing for future screening of peptides/protein libraries. Third, we demonstrate that bacteriophage-expressing a test internalization RGD peptide show enhanced uptake into MCF-7 breast cancer cells as compared to the control bacteriophage particles. Finally, we are in the process of constructing peptide and protein libraries in the bacteriophage display format. These libraries of recombinant particles will then be screened against breast cancer cells to identify potentially new peptides and proteins that are selectively endocytosed. Constructs showing enhanced uptake will be sequenced, evaluated, and compared to normal cells for their specificity in targeting only cancer cells.

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I. INTRODUCTION

Advances in cancer treatment are limited by our ability to specifically target tumor cells. This research project involves the development of a novel non-biased targeting system based on a genetic screen for identifying tumor cell targeting sequences. This screen utilizes a multifunctional targeting vector based on a modified lambda bacteriophage display system expressing protein D-fusion proteins expressed on the λ capsid surface. If the bacteriophage particles are internalized by endocytosis, the λ DNA within the particle is able to confer G418 resistance because of a Not-I buried plasmid DNA encoding the gene for G-418 resistance driven by a mammalian SV40 promoter. Thus, stable cell lines containing the integrated DNA can be recovered as plasmid vector and sequenced to determine the nature of the targeting sequence. These bacteriophage-expressing recombinatorial peptides/proteins on their surface are internalized which then allows G-418 drug resistance when incorporated into the genome of infected cells. The long-term goal is to use the screen to identify targeting sequences that are endocytosed by breast cancer cells and survive G-418 drug resistance. Surviving breast cancer cells will be expanded and their DNA will be extracted to rescue the plasmid coding for the internalized peptide sequence. Rescue plasmids will be tested for internalization in a second round by immunofluorescence. Constructs showing enhanced uptake will be sequenced and evaluated for their use in targeting breast cancer cells.

II. BODY

During the first year we have focused on validating and optimizing the different components and protocols needed to develop the bacteriophage display screening system. Accordingly, we have completed the proposed work as originally proposed in the statement of work and below we describe these research accomplishments:

Specific Aim #1 Test whether the λ display vector expressing the RGD polypeptides on its surface can be specifically internalized, integrated, and recovered from breast cancer cells.

Task#1 Generate phage particles for both wild type and RGD-expressing phage λ phage.
We constructed and generated 2 different λ bacteriophage DNA constructs. One of these constructs, the control targeting vector (λE) expresses an extra copy of protein D on its surface. The second construct (λE-RGD) expresses a cyclic RGD peptide-protein D fusion protein. DNA from λE and λE-RGD bacteriophage was prepared using plate lysis and an ion exchange chromatography. DNA sequencing was used to confirm that both DNA vectors contained the correct DNA encoding the expected fusion proteins as shown in the schematic (Fig. 1). In particular, λE and λE-RGD contains several important features. First, both encode an extra copy of the protein D coat λ protein (1, 2). Second, peptides expressed on the surface can be detected immunologically with an anti-myc antibody (since they are expressed as a fusion with a myc epitope tag). Third, the region of DNA encoding both the D coat fusion protein and drug selectable markers (mammalian and bacterial) are within a plasmid that can be released and recovered by *Not-I* restriction enzyme digestion from λ -integrated mammalian genomic DNA as described (3, 4)

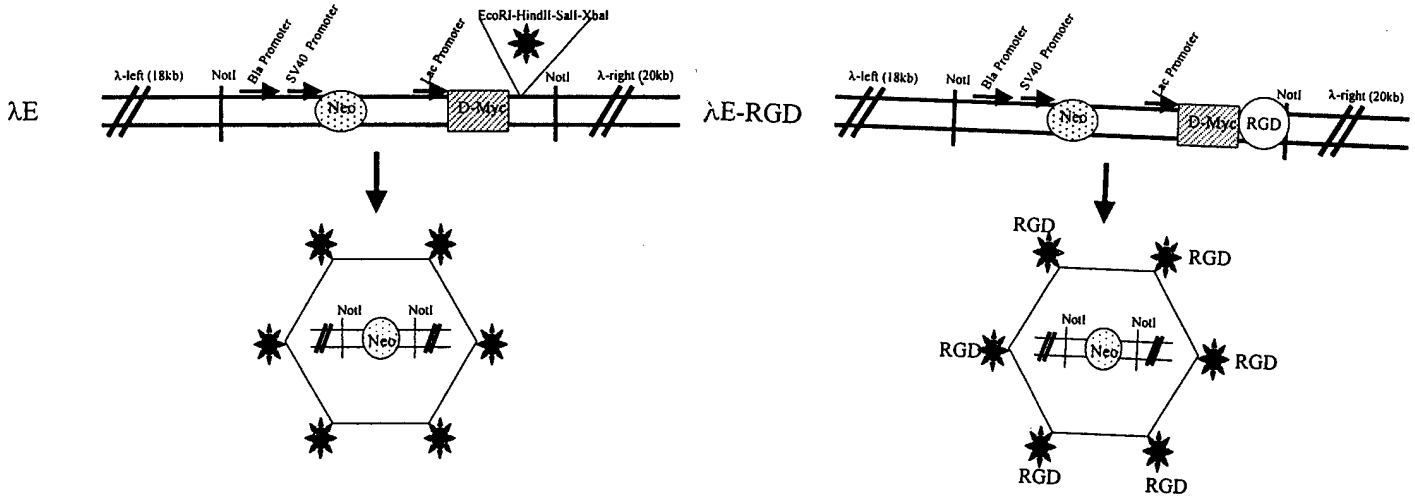

Fig. 1 Schematic of λE and λE-RGD expressing vectors
The vectors are double stranded λ bacteriophage that contain a NotI-buried plasmid. The regions encoding both the protein D -fusion protein and drug selectable markers are within the plasmid that can be released and recovered by Not-I restriction enzyme digestion. In the case of λE-RGD, a cyclic peptide (5) is fused to the carboxyl-terminal region of protein D.

Once the integrity of these DNAs was established, we prepared phage particles from λE and λE-RGD expressing bacteriophage. These bacteriophage particles were shown previously to express the new protein-D fusion protein on their surface as determined immunologically with the anti-myc antibody. Several different purification schemes were tested to obtain sterile, high quality, bacteriophage particles. Initial experiments using the bacteriophage particles, demonstrated that they were toxic and resulted in cell killing. One obstacle that was not originally foreseen was the contamination of protein particles with bacterial toxins. Thus, we have developed a purification scheme to eliminate these contaminating components. From these experiments we generated a purification scheme whereby λE and λE-RGD expressing bacteriophage were precipitated by two rounds of PEG precipitation and sterilized by passing the bacteriophage particle through a 0.2 um filter. Phage titering experiments showed that identical number of phage particles could be generated from each of two vectors using this protocol. These particles were used in sterility and cell toxicity studies and found to show significant toxicity even after 24 hours of incubation.

Task #2 and #3 Test and optimize wild type and RGD-expressing λ display phage particles for uptake into MCF-7 cells.

To initiate studies on the uptake of the λ display particles in mammalian cells, we first determined the optimum amount of Geneticin (G418) needed in the selection procedure. By performing a routine dose-response killing curve, we found that a concentration of 600 μg/ml was just above the threshold of drug needed to kill the MCF-7 cells. Next we tested whether the bacteriophage λ DNA containing the drug selectable marker cassette could confer G-418 resistance. In order to do this we purified λE and λE-RGD bacteriophage DNA by using plate lysis and a standard λ DNA purification kit (Bio101). Using this DNA we tested the ability of this DNA to yield G-418 resistant colonies. In these studies 200 ng, 500 ng and 1 μg was applied to MCF-7 cells in culture using the Fugene transfection reagent for 24 hours. After this

period of time, the cells were washed and then subjected to G-418 selection 24 hours later. Several different concentrations of DNA were transfected into 100 mm^2 dishes of MCF-7 cells. Fugene reagent (Roche) was used for transfection to deliver the DNA to these cells. By 12 days after G-418 selection, individual resistant colonies were found. If at the lowest DNA concentration, large numbers (greater than 200 colonies) of G418-resistant MCF clones were detected.

Now that the conditions of selection were optimized we used bacteriophage particles from λE and λE-RGD for uptake experiments. Initial experiments using low numbers of recombinant particles (10^5 particles) were unsuccessful. In subsequent experiments we tested much higher levels of bacteriophage particles and were able to generate G418-resistant cell lines (Table I)

Construct	Phage particle number used	G418 resistant MCF-7 colonies
-	0	0
λE	10^5	0
λE	10^6	0
λE	10^7	2
λE	10^8	14
λE-RGD	10^5	0
λE-RGD	10^6	7
λE-RGD	10^7	18
λE-RGD	10^8	43

Table I Generation of G418-expressing MCF-7 cells using λE and λE-RGD bacteriophage targeting vectors. The designated number of bacteriophage particles was added for 12 hours, washed off and subjected to G418-selection.

Based on these results, we are seeing an enhanced uptake of the λE-RGD as compared to λE bacteriophage particles. Current studies are aimed at reducing the background that we are seeing with the control vector at these phage concentrations. In particular we testing whether background levels of bacteriophage particles uptake is due to λ DNA uptake directly in the absence of the coat proteins. We are also testing whether treatment of the phage particle preparations with *DNAse-I* may eliminate the potential for DNA contamination. Another area that we are pursuing is that of altering the length of time in the uptake phase incubation with the hope that shorter times will reduce non-specific uptake of the λE particles.

Task #4 Optimize plasmid recovery from MCF-7 cells

A test system to optimize recover of the expression vector from cells is using the cell lines derived from G418-resitant colonies. Here colonies are isolated and expanded to a 100 mm^2 dishes. Using a commercially available extraction kit (Life technologies), we have prepared genomic DNA from 8 of these lines. Using this DNA we digested with *Not1*, phenol-chloroform extracted and then performed a ligation reaction. Using this ligation mixture, we transformed highly competent *E. coli* (Stratagene). From these experiments we found that we were able to recover the targeting vector from MCF-7 cells as has been shown for other expression cloning experiments (3, 4). Thus these set of experiments were straight-forward and successful.

Specific Aim #2 Identify peptide sequences from a λ display recombinatorial library that allow efficient phage internalization into breast cancer cells.

Task#1 Construct a recombinatorial peptide λ display phage library.
This aim was originally proposed for months 7-10. We have utilized a double-stranded oligonucleotide approach for generation of this peptide library. Briefly we have synthesized two synthetic pieces of 5'-GAG GAA TTC GGG TGT (NNK)10-TGT-GGC-ACT-TGA-TCT-AGA –GAG-3' and 5'- CA-CCG-TGA-ACT-AGA-TCT-CTC-3'. Using this reaction we utilized 50 ng and performed 30 cycles of PCR as described (6). Two oligonucleotides were synthesized and utilized in a PCR reaction. Following PCR the DNA was restricted with EcoR1 and Not1 restriction enzymes. Several different concentrations of double-stranded annealed oligonucleotides were ligated with a constant amount of *EcoR1-Not1* lambda phage. Following ligation at 16° C the mixture was utilized with *in vitro* packaging extracts. These packaged lysates were then utilized for infection in phage titering.

Amount of DNA fragment	Plaque-forming Units
0 ng	0
50 ng	233
100 ng	267
200 ng	456

Table II. Low complexity of recombinatorial λE libraries.
The indicated amount of PCR generated fragments were ligated with *EcoR1-Not* cut λE vector. After 16 hours the DNA, was packaged with and used to infect LE393 *E. coli* cells. The following is a representative data from two experiments.

Unfortunately, the peptide libraries are of poor complexity. The titer at the moment is not sufficient to create the libraries of peptides needed. Several additional attempts will attempt to create these libraries by directly annealed half sites of complex mixtures of these annelaed double-stranded oligonucleotides.

Construct a λ display MCF-7 cDNA library (Specific aims #3-Task 1).
Because of this we have moved forward to utilizing proteins derived from cDNAs from breast cancer cells. Although this work was not supposed to occur until much later (between months 12 -24) in the project, we have started it much earlier for several reasons. The ability to generate cDNA libraries offers a higher chance of success. We have also modified the parental vector by changing the polylinker and moving the *Not-I* site to a different location within the plasmid to facilitate larger inserts of cDNA

III. KEY RESEARCH ACCOMPLISHMENTS

We now have preliminary evidence that our hypothesis that this modified system is capable of delivering a plasmid to express a drug selectable marker within cells. These results are extremely exciting but several additional control experiments are still in progress. First the need to eliminate small amounts of lambda DNA that is missing its coat protein is still required. Our research goals involve identifying and characterizing proteins/peptide targeting sequences that

could be used in the specific delivery of chemotherapeutic agents to breast cancer cells. In order to achieve these goals, we have developed a novel targeting system based on a genetic screen using a modified, multifunctional λ bacteriophage vector. In the first year, we have developed, optimized and validated several different components of this bacteriophage display system. First, we confirmed the integrity of our test constructs, and produced both DNA and sterile bacteriophage-expressing fusion proteins on the capsid surface for internalization studies. Second, we optimized the recovery of the integrated bacteriophage DNA containing the plasmid allowing for future screening of peptides/protein libraries. Third, we demonstrate that bacteriophage-expressing a test internalization RGD peptide show enhanced uptake into MCF-7 breast cancer cells as compared to the control bacteriophage particles. Finally, we are in the process of constructing peptide and protein libraries in the bacteriophage display format. These libraries of recombinant particles will then be screened against breast cancer cells to identify potentially new peptides and proteins that are selectively endocytosed. Constructs showing enhanced uptake will be sequenced, evaluated, and compared to normal cells for their specificity in targeting only cancer cells.

IV. REPORTABLE OUTCOMES

None at this time.

V. CONCLUSIONS

We have shown poof of principal of our genetic screen based on a novel targeting system a genetic using a modified, multifunctional λ bacteriophage vector. In the first year, we have developed, optimized and validated several different components of this bacteriophage display system. First, we confirmed the integrity of our test constructs, and produced both DNA and sterile bacteriophage-expressing fusion proteins on the capsid surface for internalization studies. Second, we optimized the recovery of the integrated bacteriophage DNA containing the plasmid allowing for future screening of peptides/protein libraries. Third, we demonstrate that bacteriophage-expressing a test internalization RGD peptide show enhanced uptake into MCF-7 breast cancer cells as compared to the control bacteriophage particles. Finally, we are in the process of constructing peptide and protein libraries in the bacteriophage display format. Future experiments are directed at screening these libraries of recombinant particles to identify potentially new peptides and proteins that are selectively endocytosed. Constructs showing enhanced uptake will be sequenced, evaluated, and compared to normal cells for their specificity in targeting only cancer cells.

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